

Molecular Basis of CIB Binding to the Integrin α_{IIb} Cytoplasmic Domain*

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William T. Barry[‡], Christel Boudignon-Proudhon[‡], David D. Shock[‡], Andrew McFadden[‡],
Jonathan M. Weiss[‡], John Sondek^{‡§¶}, and Leslie V. Parise^{‡¶||**}

From the Departments of [‡]Pharmacology, [§]Biochemistry and Biophysics, ^{||}Center for Thrombosis and Hemostasis, and [¶]Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

Integrin adhesion receptors appear to be regulated by molecules that bind to their cytoplasmic domains. We previously identified a 22-kDa, EF-hand-containing protein, CIB, which binds to the α_{IIb} cytoplasmic tail of the platelet integrin, $\alpha_{\text{IIb}}\beta_3$. Here we describe regions within CIB and α_{IIb} that interact with one another. CIB binding to α_{IIb} cytoplasmic tail peptides, as measured by intrinsic tryptophan fluorescence, indicates a CIB-binding site within a hydrophobic, 15-amino acid, membrane-proximal region of α_{IIb} . This region is analogous to the α -helical targets of other EF-hand-containing proteins, such as calcineurin B or calmodulin. A homology model of CIB based upon calcineurin B and recoverin indicated a conserved hydrophobic pocket within the C-terminal EF-hand motifs of CIB as a potential integrin-binding site. CIB engineered to contain alanine substitutions in the implicated regions retained wild type secondary structure as determined by circular dichroism, yet failed to bind α_{IIb} in 11 of 12 cases, whereas CIB mutated within the N terminus retained binding activity. Thus, specific hydrophobic residues in the C terminus of CIB appear necessary for CIB binding to α_{IIb} . The identification of essential interacting regions within α_{IIb} and CIB provides tools for further probing potential interrelated functions of these proteins.

Integrins are heterodimeric transmembrane receptors that support adhesion to extracellular proteins and mediate bidirectional cellular signaling. The major integrin adhesion receptor on platelets is $\alpha_{\text{IIb}}\beta_3$, or glycoprotein IIb-IIIa, which functions to support platelet aggregation and clot retraction. In resting platelets, $\alpha_{\text{IIb}}\beta_3$ is present in a low affinity state for its major ligand, plasma fibrinogen. When platelets become activated by various agonists, “inside-out” signaling results in the rapid activation of $\alpha_{\text{IIb}}\beta_3$ to a high affinity state. Ligand binding to activated $\alpha_{\text{IIb}}\beta_3$ then stimulates “outside-in” signaling, resulting in the recruitment of signaling molecules, further platelet activation, and cytoskeletal rearrangements. Although the mechanism of this bi-directional integrin signaling is unclear, it has been proposed to involve the binding of specific molecules to the integrin α and β cytoplasmic tails (reviewed in Refs. 1 and 2).

The cytoplasmic tails of most integrin α and β subunits are relatively short (from 20 to 70 amino acids). Although β subunits are generally more conserved than α subunits, the α subunits contain conserved N-terminal membrane-proximal elements with more varied C termini (3). The cytoplasmic tails of the $\alpha_{\text{IIb}}\beta_3$ integrin have been shown to form complexes in solution (4), whereas mutations in either the α or β cytoplasmic domain that are predicted to disrupt this interaction result in integrin activation (5). Other manipulations of the α and β cytoplasmic domains that also result in integrin activation include the disruption of a β -turn in the α_{IIb} cytoplasmic domain by the substitution of alanines for 2 consecutive prolines (6) and the complete truncation of the β_3 cytoplasmic domain or deletion of 7 membrane-proximal amino acids, which support the concept of membrane-proximal amino acids that are required to maintain the integrin in a default low affinity state (7).

Furthermore, multiple proteins have been identified that bind to integrin cytoplasmic domains, the binding of which may serve to ultimately regulate integrin function. Proteins reported to bind to the β_3 cytoplasmic domain include talin (8), Shc (9), β_3 -endonexin (10), myosin (11), and Syk (12). Both talin (8) and a protein that we previously identified, termed CIB (13, 14), have been described as binding to the cytoplasmic domain of α_{IIb} .

CIB (also CIB1, SIP2–28) is a 22-kDa protein that is present in platelets but also widely distributed (14). CIB binding to the α_{IIb} cytoplasmic domain has been detected in multiple assays, including the yeast two-hybrid system (13), enzyme-linked immunosorbent assay (13), isothermal titration calorimetry (ITC)¹ (14), intrinsic tryptophan fluorescence (ITF) (the present study and Ref. 14), surface plasmon resonance (15), and co-immunoprecipitation with $\alpha_{\text{IIb}}\beta_3$ (15, 16). One study has suggested that CIB binds preferentially to the activated conformation of $\alpha_{\text{IIb}}\beta_3$, implicating a role for CIB in outside-in signaling (15). In another study it was reported that CIB binds to Rac3 and that coexpression of active Rac3 and CIB in Chinese hamster ovary cells also expressing $\alpha_{\text{IIb}}\beta_3$ results in enhanced $\alpha_{\text{IIb}}\beta_3$ -mediated cell adhesion and spreading on fibrinogen (17). Finally, a separate study recently implicated CIB in inside-out integrin activation in platelets (16).

CIB contains at least two functional C-terminal EF-hand domains, as well as two N-terminal EF-hand-like sequences also likely to be functional. CIB is homologous to other EF-hand-containing proteins such as the regulatory molecules, calcineurin B (CnB, 58% homology) and calmodulin (CaM, 56%

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** To whom correspondence should be addressed: Dept. of Pharmacology, CB 7365, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-2238; Fax: 919-966-5640; E-mail: parise@med.unc.edu.

¹ The abbreviations used are: ITC, isothermal titration calorimetry; CD, circular dichroism; ITF, intrinsic tryptophan fluorescence; CnA, calcineurin A; CnB, calcineurin B; CaM, calmodulin; Ac, acetylated; mAb, monoclonal antibody; PDB, Protein Data Bank.

homology) (13). Members of the EF-hand superfamily of proteins undergo conformational changes upon Ca^{2+} binding to the helix-loop-helix structures that regulate interaction with target proteins (18). Thus, it is not unexpected that CIB binds in a calcium-dependent manner to the α_{IIb} cytoplasmic tail, as we previously demonstrated by ITC (14). Like other close relatives in the EF-hand superfamily, CIB contains an N-terminal myristoyl group (19), which is predicted to promote an association of CIB with the cell membrane and which points to CIB as a member of the Ca^{2+} -myristoyl switches, a newly recognized family of proteins that regulate effector protein function at the membrane in a calcium-dependent manner (20).

Here we identify a limited region of the α_{IIb} cytoplasmic domain, including a portion of the predicted membrane-spanning region, and critical hydrophobic residues therein that mediate CIB binding. In addition, we find that CIB is detected exclusively in membrane-containing fractions of N_2 -cavitated platelets, consistent with a potential *in vivo* interaction of CIB with the membrane-proximal region of α_{IIb} . Furthermore, we identify a hydrophobic pocket within the C-terminal EF-hand/ Ca^{2+} -binding motifs of CIB that appears necessary for binding to the α_{IIb} integrin. The identity of these regions suggests a largely hydrophobic interaction between α_{IIb} and CIB and will facilitate the future development of molecular tools to further understand the structure-function relationship of CIB to $\alpha_{IIb}\beta_3$ signaling in platelets.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—N-terminally acetylated peptides corresponding to the C-terminal portion of the α_{IIb} integrin (LVLAMWVKVGFKRNR-PPLEEDDEEGQ) were synthesized and purified using high performance liquid chromatography by the Protein Chemistry Laboratory of the University of North Carolina (Chapel Hill, NC).

Production of Recombinant CIB—For ITF binding studies, recombinant CIB was expressed and purified as previously described (14). Briefly, human CIB was expressed in *Escherichia coli* as a fusion protein with glutathione S-transferase in pGEX-2T (APBiotech). Cells were lysed in buffer A (phosphate-buffered saline, pH 7.5, containing 5 mM dithiothreitol, 20 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM phenylmethylsulfonyl fluoride) by passage through a French press (SLM Instruments, Urbana, IL). Cell lysate was clarified by centrifugation, loaded onto a glutathione-Sepharose 4B column (APBiotech), and eluted in buffer containing 10 mM reduced glutathione. The eluate was incubated with bovine α -thrombin (APBiotech) at 10 units/mg of protein, for 2–4 h at ambient temperature. CIB was further purified by gel exclusion using a HiPrep 16/60 S-200 column (Amersham Biosciences) followed by passage over a glutathione-Sepharose 4B (Amersham Biosciences) column to remove residual glutathione S-transferase. A final purification of recombinant CIB was performed over a Resource Q column (Amersham Biosciences) eluted in a linear gradient of 0–500 mM NaCl. Fractions containing pure CIB were concentrated by ultrafiltration and stored at -80°C . CIB purity was determined to be >99% on Coomassie Brilliant Blue-stained polyacrylamide gels.

For CIB used in circular dichroism (CD) studies, wild type and mutant CIB constructs were expressed in BL21 cells using the pPROEX HTa vector (Invitrogen). For protein extraction, induced cells were resuspended in lysis buffer (20 mM NaPO_4 , pH 8.0, 300 mM NaCl, 10 mM imidazole, 1:200 protease inhibitor mixture (Calbiochem), 0.1 M phenylmethylsulfonyl fluoride) and lysed in a French press as above. For protein purification, the samples were separated on nickel-nitrilotriacetic acid-agarose columns (Talon resin, CLONTECH) and His-tagged labeled proteins were eluted with 250 mM imidazole. The purified proteins were stored at -80°C in 10% glycerol.

Intrinsic Tryptophan Fluorescence—ITF was measured using a PerkinElmer Life Sciences LS-50B luminescent spectrophotometer. The fluorescent spectra of α_{IIb} cytoplasmic domain peptides in ITF buffer (1–10 μM in 20 mM Hepes, pH 7.5, 137 mM NaCl, 5 mM CaCl_2) were measured at 20°C . The excitation wavelength was set at 295 nm with a slit width of 10 nm, and emission was measured from 310 to 420 nm. Trp^{988} emission was maximal at ~ 350 nm; in the presence of saturating concentrations of CIB, the ITF of α_{IIb} increased $\sim 75\%$ at λ_{max} . Saturation binding isotherms were generated by sequential additions of 5- μl aliquots of 0.5 mM CIB to 2 ml of ITF buffer containing 10

μM α_{IIb} peptide and plotting the change in fluorescence (ΔF) against the molar ratio of CIB to α_{IIb} . The stoichiometry of binding was determined from the initial slope of the percentage of saturation of ΔF plotted against the concentration of CIB, whereas a dissociation constant (K_d) was calculated by extrapolating the amount of free CIB from the percentage of saturation of ΔF .

Platelet Preparation, N_2 Cavitation, and CIB Immunoprecipitation—Washed platelets were prepared as described previously (14). Platelets were centrifuged at $750 \times g$ and resuspended in cavitation buffer (50 mM Hepes, pH 7.5, 1 mM MgCl_2 , 500 μM CaCl_2 , 1 mM dithiothreitol, 10 $\mu\text{g}/\text{ml}$ aprotinin, and 10 $\mu\text{g}/\text{ml}$ leupeptin, 4°C) to a concentration of $\sim 2 \times 10^9$ platelets/ml. Suspended platelets were disrupted by nitrogen cavitation using a cell disruption bomb (Parr, Molina, IL) at 1500 p.s.i., three times for 20 min. Cavitated platelets were collected and centrifuged at $100,000 \times g$ for 1 h at 4°C . Supernatants were collected as the cytosolic fraction. Pellets were resuspended with cavitation buffer, layered over 30% sucrose, and centrifuged at $100,000 \times g$ for 1 h at 4°C . The interphase of proteins was recovered as the purified membrane fraction. Cytosol and membrane fractions were solubilized in sample buffer containing 2% SDS and analyzed by SDS-PAGE. CIB was also immunoprecipitated from membrane and cytosol fractions, first by pre-clearing with control IgG and GammaBind G (Amersham Biosciences), followed by immunoprecipitation with 10 $\mu\text{g}/\text{ml}$ CIB-specific mAb, UN2, or isotype-matched control IgG1. Samples were separated on 4–12% Novex pre-cast gels, transferred to polyvinylidene difluoride, and visualized by ECL (APBiotech).

Homology-based Model of CIB—A homology-based model of calcium-occupied CIB was generated with Modeler from Insight II (Molecular Simulations, San Diego, CA) using structures of other calcium-binding proteins homologous to CIB. Calcineurin B (subunit B of Protein Data Bank (PDB) identification code 1TCO) (21) and recoverin (PDB identification code 1JSA) (22) were identified as templates for the CIB sequence using the Find_Structures option from the Profiles-3D application in Insight II. The CIB sequence was aligned to the calcium-binding proteins using the Align2D option from Modeler, and the alignment was compared with results from other alignment programs. Models of CIB were built using Modeler from the Homology module in Insight II using both templates as reference structures. To construct an 8-amino acid insert in the N terminus and a 5-amino acid insert in the C terminus, multiple loops were generated for the model. All homology models constructed were evaluated using the Profiles-3D Verify function. The homology model identified as being most compatible with the CIB sequence was used for further analysis.

Yeast Two-hybrid System—CIB was previously identified in a yeast two-hybrid screen as an interacting protein with the α_{IIb} integrin cytoplasmic tail (13). QuikChange site-directed mutagenesis (Stratagene) of CIB in pGAD10 was used to generate 14 single, double, and triple alanine substitutions in the CIB sequence. The CIB constructs were transformed into yeast strain Y187 and mated with Y190 cells containing the α_{IIb} cytoplasmic tail in the pGBT9 vector (CLONTECH). For each mutant, three colonies were chosen, grown in selection media (Leu $^-$, Trp $^-$ SD media), and assayed for β -galactosidase activity with *O*-nitrophenyl- β -D-galactopyranoside (protocol PT3024-1, CLONTECH). Expression of the CIB- and α_{IIb} -GAL4 fusion proteins was confirmed by immunoblotting yeast lysates with monoclonal antibodies against the activation domain or the DNA-binding domain of GAL4 (CLONTECH).

Circular Dichroism—The secondary structure of recombinantly expressed wild type and mutant CIB were verified by CD analysis, using a Pistar-180 CD Spectrometer (Applied Photophysics, Leatherhead, UK). Proteins were first dialyzed into a buffer containing 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , pH 6.8, and 250 μM CaCl_2 , and protein concentrations were determined via BCA protein assay (Pierce). The CD spectra were read from 190 to 260 nm at 20°C . Secondary structure was predicted from the CD spectrum using the program CDstr (23).

RESULTS

CIB Interaction with α_{IIb} by Intrinsic Tryptophan Fluorescence—We previously reported that CIB bound to the cytoplasmic tail of the α_{IIb} integrin as detected by yeast two-hybrid screening (13) and by ITC (14). To identify the binding domain for CIB within the integrin tail, we measured CIB binding to various α_{IIb} integrin peptides via ITF. Because the 191-amino acid sequence of CIB does not contain any tryptophan residues, whereas the integrin cytoplasmic tail contains Trp^{988} , CIB binding could be characterized by changes in the ITF of α_{IIb}

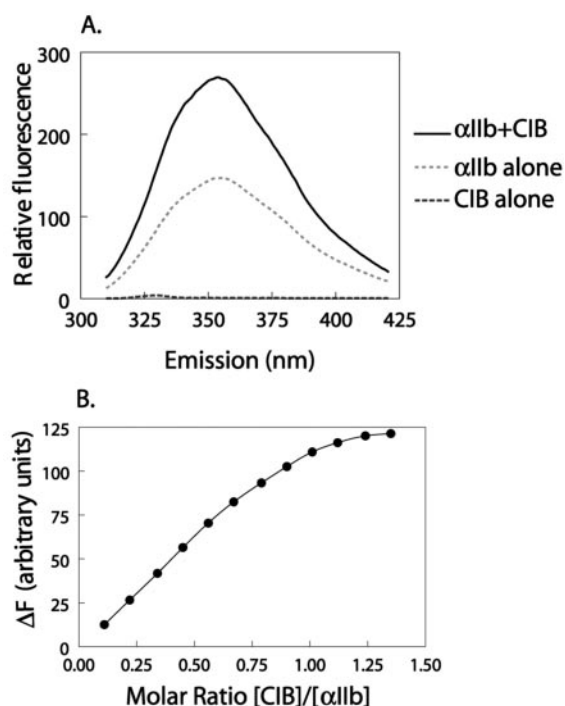


FIG. 1. Measurement of CIB binding to the cytoplasmic domain of the α_{IIb} integrin by ITF. A, shown are the fluorescence spectra of CIB alone (12.5 μ M), wild type α_{IIb} peptide (10 μ M, Ac-LVLAMWKVGGFFKRNRPPEEDDEEGQ) alone, or α_{IIb} peptide (10 μ M) with a saturating concentration of CIB (12.5 μ M). B, CIB binds wild type α_{IIb} peptide with a K_d of ~ 0.3 μ M and a stoichiometry of 1:1. The change in fluorescence at $\lambda_{max} = 350$ nm is plotted against the molar ratio of CIB to α_{IIb} peptide. Titrations were performed as described under "Experimental Procedures."

upon titration with CIB. The emission spectrum of the full-length, wild type α_{IIb} cytoplasmic tail exhibited a λ_{max} at ~ 350 nm (Fig. 1A). Saturating concentrations of CIB resulted in an $\sim 75\%$ increase in fluorescence intensity at λ_{max} . CIB addition also resulted in a very slight blue-shift (< 2 nm) of the tryptophan emission. A saturation binding isotherm representing the interaction of CIB with the wild type α_{IIb} cytoplasmic tail, as detected by ITF (Fig. 1B), indicated a 1:1 stoichiometry of binding and a dissociation constant (K_d) of 0.3 μ M, similar to values previously obtained by ITC (14).

Because the membrane-proximal region of α_{IIb} is hydrophobic and determined to be α -helical (24) like other targets for EF-hand-containing proteins, such as the α -helical region of calcineurin A (CnA) that binds to CnB, we predicted that this region of α_{IIb} might be critical for binding to CIB. To test this hypothesis, a series of truncated α_{IIb} peptides were examined by ITF for their ability to bind CIB. Peptides lacking the last 8 (Leu⁹⁸³–Leu¹⁰⁰⁰) or 11 (Leu⁹⁸³–Arg⁹⁹⁷) C-terminal amino acids retained the CIB-induced change in fluorescence (Fig. 2A), indicating an interaction with CIB and suggesting that CIB binding activity resides within in the N-terminal, membrane-proximal portion of the α_{IIb} tail. The 11-amino acid deletion peptide (Leu⁹⁸³–Arg⁹⁹⁷) retained a K_d for CIB similar to the full-length peptide (determined by titration as in Fig. 1B; data not shown). To further confirm this observation, we asked whether a peptide containing only the charged C-terminal amino acids of α_{IIb} (Ac-LEEDDEEG) had any detectable CIB binding activity. Because this peptide lacked a tryptophan necessary for ITF, we tested its ability in a competition assay to inhibit the CIB-induced ΔF of the full-length α_{IIb} cytoplasmic domain peptide. However, the C-terminal peptide failed to attenuate the CIB-induced ΔF (data not shown.) These data further support the conclusion that the membrane-proximal,

N-terminal portion of the α_{IIb} cytoplasmic tail contains the binding site for CIB.

The importance of specific hydrophobic residues within the CIB-binding region of α_{IIb} was probed by alanine substitution. Substitution of alanine for Leu⁹⁸³, Phe⁹⁹², or Phe⁹⁹³ resulted in a complete loss of the CIB-induced ΔF , implicating these residues as essential for CIB binding (Fig. 2B). Additionally, a truncated Trp⁹⁸⁸ \rightarrow Tyr peptide (Ac-LVLAMYKVGFFKRNRR) was unable to attenuate the CIB-induced ΔF of the truncated α_{IIb} peptide (Leu⁹⁸³–Arg⁹⁹⁷), suggesting that Trp⁹⁸⁸ is critical for CIB binding to the α_{IIb} integrin (Fig. 2C). A minimal CIB binding sequence in α_{IIb} , extending from residue Leu⁹⁸³ to Arg⁹⁹⁷, is summarized in Fig. 2D. Of note, this predicted CIB-binding region extends by ~ 6 amino acids (Leu⁹⁸³–Trp⁹⁸⁸) to within the putative membrane-spanning region of α_{IIb} (3).

Membrane Localization of CIB—Because the CIB binding site appears to extend into the predicted membrane-spanning region of α_{IIb} , we asked whether CIB distributes with the membrane fraction derived from nitrogen-cavitated platelets. We found that CIB fractionates exclusively with platelet membranes, as detected by immunoblotting both membrane and cytosolic fractions with the CIB-specific mAb, UN2 (Fig. 3). To further confirm a lack of CIB in the cytosolic fraction, CIB in each fraction was concentrated by immunoprecipitation, followed by immunoblotting, but, again, no CIB was detected in the cytosolic fraction (Fig. 3). CIB therefore appears to be membrane-associated in platelets, increasing the likelihood of a potential *in vivo* interaction between CIB and the α_{IIb} integrin subunit.

Comparison of CIB to Calcineurin B—In predicting the sites within CIB that bind to α_{IIb} , we first noted that the primary sequence of CIB best fit the structures of CnB (21) and recoverin (22). Furthermore, many EF-hand domains bind amphipathic α -helical regions through complementary hydrophobic surfaces (25), and we noticed a striking similarity in the amphipathic character of the α_{IIb} cytoplasmic tail that forms an α -helix (24), and the C-terminal portion of CnA that binds CnB. Similarly, many of the hydrophobic residues within the C-terminal EF-hand domains of CnB implicated in binding CnA are highly conserved in CIB (13), suggesting a common mode of complex formation. Therefore, the structure of CnB bound to CnA (Fig. 4A) together with a multiple sequence alignment of proteins containing EF-hand domains (only CIB and CnB are shown in Fig. 4B) were used to identify highly conserved residues within CIB most likely necessary for productive interaction with α_{IIb} . These residues were mutated and the functional consequences assessed to identify residues potentially critical for CIB binding to α_{IIb} .

Yeast Two-hybrid Analysis and CD Spectroscopy—CIB mutants were generated with alanine substitutions by site-directed mutagenesis. Although most mutations were of hydrophobic residues based on the model of CIB as compared with CnA and CnB (Fig. 4, A and B), we also targeted two sequential arginines (residues 32 and 33) in the N terminus of CIB that had been previously implicated as part of a potential site of integrin binding (26). The ability of the CIB mutants to interact with the α_{IIb} cytoplasmic tail was analyzed in the yeast two-hybrid system (Fig. 4C). As previously reported (13), wild type CIB interacted positively with the α_{IIb} cytoplasmic tail, compared to an empty vector control. Although alanine substitutions for the sequential arginines (Arg³² \rightarrow Ala/Arg³³ \rightarrow Ala) in the N terminus of CIB had no effect on CIB binding to the α_{IIb} tail, 11 of 12 mutations to Ala and 1 to Thr of hydrophobic residues in the C-terminal EF-hands of CIB resulted in a loss of interaction. The apparent loss of interaction could not be accounted for by a loss of mutant CIB expression, as Western

FIG. 2. Residues 983–997 of α_{IIb} define a minimal binding determinant for CIB. *A*, shown is the fluorescence ($\lambda_{\max} = 350$) of $10 \mu\text{M}$ truncated α_{IIb} peptides (Leu⁹⁸³–Leu¹⁰⁰⁰ and Leu⁹⁸³–Arg⁹⁹⁷), with 0 or $20 \mu\text{M}$ CIB. *Single-letter codes* are used for amino acids. *B*, fluorescence ($\lambda_{\max} = 350 \text{ nm}$) of $1 \mu\text{M}$ α_{IIb} peptide and 0 or $2 \mu\text{M}$ CIB for both wild type and mutant α_{IIb} peptides with alanine substitutions at Leu⁹⁸³, Phe⁹⁹², and Phe⁹⁹³. *C*, Trp⁹⁸⁸ of α_{IIb} is critical for high affinity interaction with CIB. The α_{IIb} peptide (Trp⁹⁸⁸ \rightarrow Tyr; Ac-LVLAMYKVGFFKRNR) failed to compete effectively with wild type α_{IIb} (Leu⁹⁸³–Arg⁹⁹⁷) for binding to CIB, as indicated by sustained fluorescence even in the presence of up to 20-fold molar excess α_{IIb} (Trp⁹⁸⁸ \rightarrow Tyr). *D*, primary sequence of α_{IIb} cytoplasmic domain with minimal region necessary for binding CIB is highlighted in *gray* and critical hydrophobic residues are *boxed*.

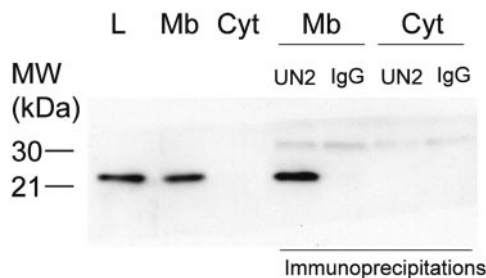
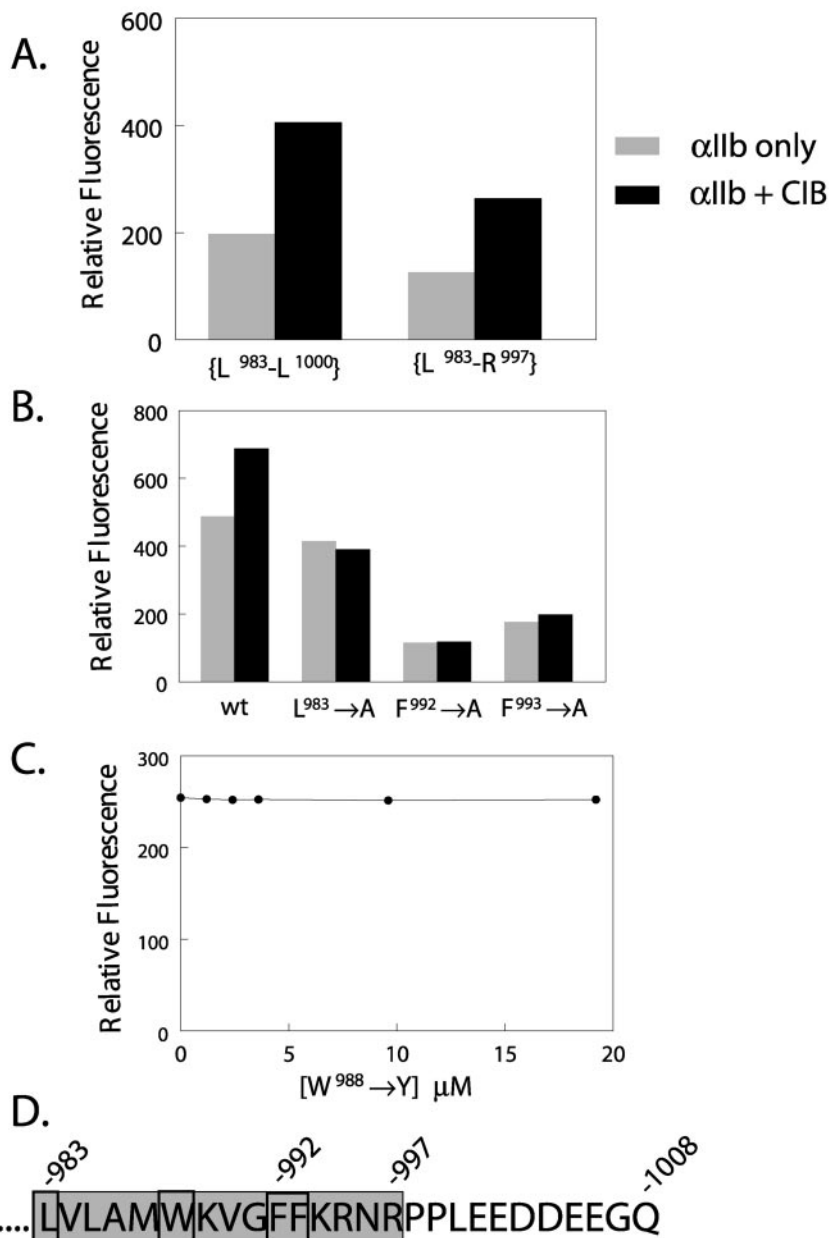


FIG. 3. CIB fractionates exclusively with platelet membranes. Nitrogen-cavitated platelets were fractionated on a sucrose cushion to separate cytosolic (*Cyt*) and membrane (*Mb*) fractions. CIB was also immunoprecipitated from the platelet fractions with either the CIB-specific mAb, UN2, or an IgG control. Fractions were separated by SDS-PAGE, transferred to Immobilon-P membrane, and blotted with UN2. *L*, total lysate.

blotting of yeast extracts demonstrated equal expression of the wild type and mutant CIB/Gal4 activation domain fusion proteins (data not shown). Moreover, the inability of most of the CIB mutants to interact with α_{IIb} could not be accounted for by

a global loss of secondary structure, as verified by CD spectroscopy (Fig. 4D). The CD spectrum of CIB predicts a secondary structure that is composed of 46% α -helix and 12% β -sheet, similar to predictions derived from the homology model of CIB (Table I). As compared with wild type CIB, most of the mutants retained substantially similar secondary structure predictions of a highly α -helical protein (Fig. 4D). The only mutants where overall secondary structure appears to be slightly affected include Phe¹¹⁵ \rightarrow Ala, Phe¹⁷³ \rightarrow Ala, and the multiple mutation of Leu¹³¹-Val-Asn-Cys-Leu¹³⁵ to Ala-Ala-Asn-Cys-Ala. Based on this analysis, the loss of CIB binding activity in most mutants is unlikely to be explained by a global change in conformation.

Homology Model of CIB—To visualize the location of these critical amino acids within a three-dimensional structure, we built a homology model of CIB from the published structures of CnB (PDB identification code 1TCO) (21) and recoverin (PDB identification code 1JSA) (22). Although CIB is also reasonably homologous to CaM, the CIB sequence fit structures of CaM poorly (PDB identification code 1CKK) (27, 28). The final homology model of CIB (Fig. 4E) contained 52% α -helix, consistent with the helical content of full-length CIB as determined by CD spectra (Table I). The residues

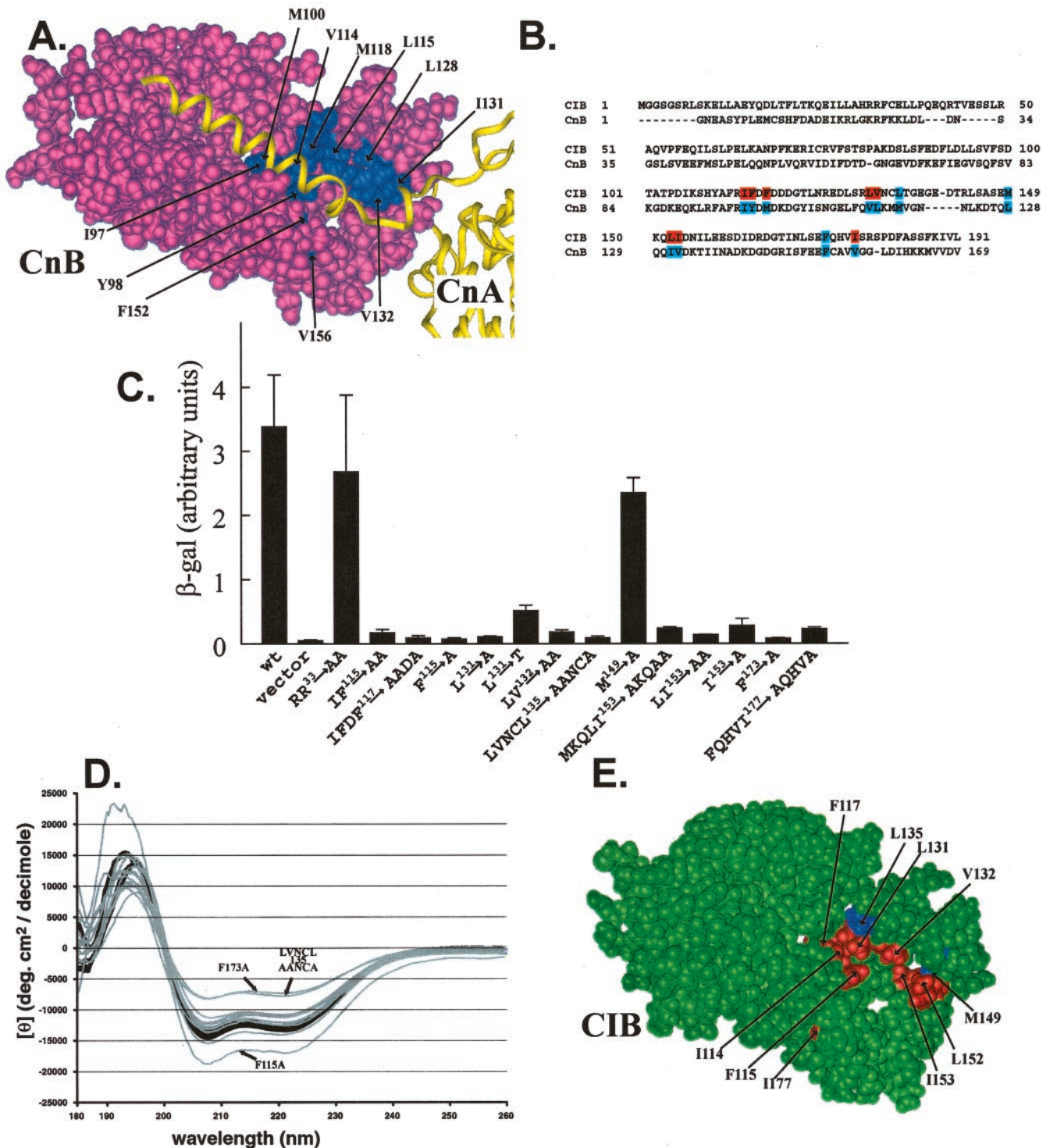


FIG. 4. Regions of CIB essential for interaction with α_{IIb} . *A*, van der Waals model of calcineurin B bound to calcineurin A (21). Highlighted in blue are hydrophobic amino acids in CnB that are within the binding pocket for CnA and are conserved in CIB. *Single-letter codes* are used for amino acids. *B*, primary sequence alignment of human CIB with bovine calcineurin B. Shown is a subset of a multiple sequence alignment of EF-hand domains, emphasizing CIB (NCBI accession no. NP_006375) and CnB (NCBI accession no. 1942334). Residues highlighted in blue in *panel A* are similarly highlighted in the primary sequence of CnB. Residues highlighted in red in the CIB sequence are those deemed critical for productive binding to α_{IIb} ; blue residues in the CIB sequence were mutated but not shown conclusively to participate in α_{IIb} complex formation (also depicted in the model of CIB in *E*). *C*, yeast two-hybrid analysis of CIB or mutant CIB fusion protein binding to the wild type (*wt*) α_{IIb} sequence (LVLAMWKVGFVKRNRPPLEEDDEEGQ) as detected by relative β -galactosidase (β -gal) activity. *D*, circular dichroism spectroscopy of wild type and mutant forms of CIB. *E*, van der Waals depiction of a homology model of CIB indicating residues highlighted in red determined to be critical for productive binding of α_{IIb} . Blue residues were mutated but were not shown conclusively to participate in α_{IIb} complex formation (Phe¹⁷³ is not visible in this orientation).

in CIB that are essential for α_{IIb} binding and that do not compromise the overall protein fold upon substitution are highlighted in red in the homology model of CIB (Fig. 4E).

DISCUSSION

We previously identified CIB in the yeast two-hybrid system as a binding partner for the cytoplasmic tail of the α_{IIb} integrin

TABLE I
Secondary structure predictions of CIB from homology model versus circular dichroism analysis

	α -Helix	β -Sheet	Turn	Random coil
	%	%	%	%
Homology model	52	3	8	37
Circular dichroism ^a	46 \pm 0.4	12 \pm 0.4	20 \pm 0.4	19 \pm 2.1

^a Secondary structure values were computed with the CDstr program from three CD spectra of wild-type CIB from 260 to 190 nm.

subunit (13) and further demonstrated that this binding occurs in a Ca^{2+} -dependent manner (14). Moreover, recent studies have provided evidence that CIB, in conjunction with Rac3, activates $\alpha_{IIb}\beta_3$ -mediated adhesion and spreading in cultured cells (17) and that CIB binding to $\alpha_{IIb}\beta_3$ may activate the soluble fibrinogen-binding function of purified and platelet-associated $\alpha_{IIb}\beta_3$ (16). Because of the potential for CIB to regulate $\alpha_{IIb}\beta_3$ integrin function in platelets, it was of interest to identify the CIB-binding region within the α_{IIb} cytoplasmic domain and the integrin-binding region within CIB. We localized the CIB binding site on α_{IIb} to within a 15-amino acid stretch that includes residues within the membrane-proximal cytoplasmic region as well as several residues within the predicted membrane-spanning region of α_{IIb} . Furthermore, four hydrophobic amino acids within this 15-amino acid sequence were shown to be critical for high affinity α_{IIb} peptide binding to CIB. Finally, we have identified an apparent hydrophobic binding pocket within CIB for the α_{IIb} cytoplasmic domain.

CIB binding to α_{IIb} cytoplasmic tail peptides was detected by an increase in ITF of these peptides. Intrinsic tryptophan fluorescence, in the presence of calcium, indicated a 1:1 stoichiometry and a K_d of 0.3 μM for recombinant CIB binding to the α_{IIb} cytoplasmic tail peptides. These values are similar to those previously reported with ITC (1:1 stoichiometry, K_d 0.7 μM (Ref. 14)), further verifying the usefulness of ITF for this study. In addition, ITF has the advantage over ITC of requiring much less protein for detection of binding interactions. The increase in α_{IIb} fluorescence is presumably because of a decrease in solvent quenching of the tryptophan emission upon formation of a CIB- α_{IIb} complex (29).

As measured by ITF, CIB binds with equal affinity to both the full-length α_{IIb} peptide (Leu⁹⁸³-Gln¹⁰⁰⁸) corresponding to the cytoplasmic tail plus 6 amino acids within the putative membrane-spanning region, as to a truncated peptide (Leu⁹⁸³-Arg⁹⁹⁷) lacking the last 11 amino acids. It is noteworthy that, although CIB binds to α_{IIb} in a membrane-proximal region that is highly conserved among α subunits (reviewed in Ref. 3), it binds exclusively to the integrin α_{IIb} subunit. In fact, the integrin subunit most homologous to α_{IIb} in this region is α_v , yet we previously observed with multiple attempts in the yeast two-hybrid system that CIB does not bind to the α_v cytoplasmic domain (13).² This can most easily be explained by a tyrosine in α_v in a position corresponding to a critical tryptophan residue (Trp⁹⁸⁸) in α_{IIb} . Indeed, the single conservative substitution of a tyrosine for tryptophan in this position of α_{IIb} causes an apparent loss of CIB binding activity (Fig. 2C). The substitution of alanine for other hydrophobic residues in the α_{IIb} cytoplasmic tail (*i.e.* Leu⁹⁸³, Phe⁹⁹², and Phe⁹⁹³) also disrupted binding to CIB. These results further substantiate the hypothesis that α_{IIb} and CIB bind primarily through hydrophobic interactions. Such a set of interactions is analogous to those seen in complexes formed with other calcium binding proteins such as CnB (21). Two of the critical residues in α_{IIb} , Leu⁹⁸³ and Trp⁹⁸⁸, are considered to be within the transmembrane domain

of the integrin (3), indicating that CIB should localize to the platelet membrane at least part of the time to interact physiologically with α_{IIb} .

Although CIB contains an N-terminal myristoylation site and was recently shown to be myristoylated in HeLa cells (19), it was unknown whether CIB associates with membranes in platelets. In nitrogen-cavitated platelets, we observed that CIB is retained exclusively with the membrane fraction, consistent with the potential for an *in vivo* interaction of CIB with α_{IIb} that involves several amino acids of α_{IIb} potentially inserted into the plasma membrane. In addition, CIB is homologous to calcium-myristoyl switch proteins such as recoverin (22). However, whether CIB actually functions in the same manner to extrude its myristoyl group with increasing Ca^{2+} concentrations and translocate to membranes is currently unknown. In any case, because CIB binds to these intramembraneous amino acids, an interesting hypothesis is suggested for integrin activation, whereby the application of an intramembraneous force to the α_{IIb} subunit alters the $\alpha_{IIb}\beta_3$ complex in a manner similar to the integrin-hinge hypothesis proposed by Hughes *et al.* (5).

The homology between CIB and the EF-hand-containing proteins, calcineurin B (58%) and calmodulin (56%), suggested that CIB might bind to the α -helical region of the α_{IIb} cytoplasmic domain by a mechanism similar to that used by other EF-hand-containing proteins. Calcium binding to a number of other EF-hand-containing proteins is known to substantially alter the tertiary structure of these proteins, exposing the binding sites for effector molecules (18), and we previously demonstrated that CIB binding to α_{IIb} was Ca^{2+} -dependent (14). Building a homology model of CIB based upon the published structures of calcium-occupied CnB and recoverin allowed us to identify a hydrophobic region in CIB that is potentially exposed on the surface of the molecule. This C-terminal hydrophobic pocket was highly conserved between CnB and CIB, and is also part of the binding domain for the catalytic subunit of calcineurin, CnA. Furthermore, the CnB binding site on CnA consists of a long, amphipathic α -helix, similar to the binding site identified on α_{IIb} (21). This evidence led us to target the hydrophobic pocket in CIB as a potential integrin-binding site. In our mutational analysis, we also targeted two arginine residues in the N terminus previously implicated as a potential site of integrin interaction (26). Circular dichroism demonstrated the percent α -helical, β -sheet, and random coil content of wild type CIB and indicated that most of the CIB mutants retain a nearly identical structure, with only three of the mutant CIB molecules deviating perceptibly in structural composition. As demonstrated in the yeast two-hybrid analysis, α_{IIb} binding to CIB is dependent upon hydrophobic residues in the C terminus of CIB, and not the charged residues mutated in the N terminus. Therefore, CIB binds to the membrane-proximal region of the α_{IIb} cytoplasmic tail through a hydrophobic interaction that is regulated by calcium occupancy of the EF-hands in CIB. Although a very recent study suggested that the extreme C terminus of CIB contains an α_{IIb} binding site, the ability of this region to interact directly with α_{IIb} was not demonstrated (16). Moreover, our mutations would suggest that the CIB binding site extends beyond and/or outside of this region to include the hydrophobic binding pocket visualized in Fig. 4E.

In conclusion, we have narrowed down the CIB binding site in α_{IIb} to an N-terminal region of the cytoplasmic domain that partially extends into the putative membrane spanning region. In addition, we have identified specific residues within a hydrophobic pocket in CIB that appear to mediate integrin binding. These results demonstrate interacting regions of CIB and

² W. T. Barry, C. Boudignon-Proudhon, and L. V. Parise, unpublished observations.

α_{IIb} and offer a potential mechanism of $\alpha_{IIb}\beta_3$ regulation by CIB. This knowledge will be applicable to future structure/function studies of CIB both *in vitro* and *in vivo*.

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